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Evaluation of the Antimicrobial Activity of Natural Animal Proteins/Peptides In Vitro

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Abstract
The objectives of this research were to validate the sensitivity and precision of an *in vitro* assay for evaluating the efficacy of antimicrobials, to evaluate the ability of natural animal proteins/peptides to kill *in vitro* antibiotic-resistant, as well as, -susceptible bacteria, and to determine the effects of key components of animal digesta (e.g., pH, mineral content, and proteolytic digestive enzymes) on the estimated antimicrobial activity of these proteins/peptides.

The minimum inhibitory concentrations (MIC) for polymyxin B (control antibiotic) were determined to be .76, .76, and .90 μg/mL for *Escherichia coli*, *Escherichia coli* (nalidixic acid-resistant), and *Staphylococcus aureus*, respectively. The intra- and inter-assay variation for MIC determination was .18 and .2%, respectively.

The natural animal proteins and peptides (lactoferrin, lactoferricin B, hen egg lysozyme, and alpha-lactalbumin LDT2) were determined in *in vitro* (acetic acid medium) to kill selected bacteria. Each of the tested proteins/peptides was active against an antibiotic-resistant (nalidixic acid) strain of *E. coli*; however, the required concentrations for antimicrobial activity were 10 to 15 times higher than that of the nonantibiotic-resistant strain. The antimicrobial activity of each protein/peptide in animal digesta fluid was 130 to 300% greater than that in the acetic acid media. Overall, the intra- and inter-assay variation values for the tested proteins/peptides was 3 and 3.4%, respectively.

The antimicrobial activity of two of the three proteins/peptides was not affected by the presence of cationic minerals. The change in pH (digesta fluid and acetic acid media) from 7 to 2 resulted in a loss of antimicrobial activity of 33% for all proteins/peptides. Therefore, the increase in antimicrobial activity associated with the digesta fluid is not related to change in H or the mineral concentration of the digesta. Based on these data, natural proteins/peptides represent potential antibiotic substitutes.

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Evaluation of the Antimicrobial Activity of Natural Animal Proteins/Peptides In Vitro

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Summary and Implications

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Introduction

With the recent restrictions of antibiotic use in food-producing animals (European Commission, Dec. 10, 1998), the greater concerns about antibiotic-resistant bacteria in both the animal and human population and the desire to reduce foodborne pathogen levels, an increasing need to develop effective but human and environmental compatible, antibiotic alternatives for the pork industry and the medical industry has arisen.

Recently, natural proteins have been identified that possess these attributes. *In vitro*, these proteins have been shown to be antimicrobial peptides (AMP) or directly form AMP upon degradation by digestive enzymes, stimulate endogenous synthesis of AMPs by the animal, and induce immune responses favorable to bacterial removal.

Proteins with these characteristics have been identified as components of milk (e.g., lactoferrin, lactoferricin, and alpha-lactalbumin), eggs (e.g., lysozyme), and animal tissues (e.g., defensins). These proteins can function as a natural bacterial barrier for the pathogen-susceptible neonate, embryo and animal cells or organs, respectively.

Some AMPs exhibit unique mechanisms for killing bacteria compared with current antibiotics used in the pork industry. These AMPs selectively bind to the outer lipid membrane of the bacterium and form blisters and pores, which eventually result in lysis of the cell and cellular death (12). AMPs also have the ability to stimulate the production of IL-1B (11). The stimulation of IL-1B would create in increase in chemotaxis of the neutrophils to that area. These neutrophils contain AMPs produced from the animal, which would serve as a secondary source of AMPs for the host.

Based on these *in vitro* data, it is hypothesized that the feeding of these natural proteins results in the enteric production of antimicrobial peptides, which function as effective antibiotics via the direct antimicrobial activity of the peptides, and the peptide's indirect enhancement of the immune response of the animals. Because of their unique mechanism for killing bacteria, it also is believed that the AMPs or their precursor may be effective in killing antibiotic-resistant, as well as antibiotic-sensitive, bacteria. However, the antimicrobial efficacy of these AMPs *in vivo*, particularly in the animal digestive tract, has not been defined.

The objectives of this research were to validate the sensitivity and precision of an *in vitro* assay for evaluating the efficacy of antimicrobials, to evaluate the ability of natural animal proteins/peptides to kill *in vitro* antibiotic-resistant, as well as, susceptible bacteria, and to determine the effects of components of animal digesta

(e.g., pH, mineral content, and proteolytic digestive enzymes) on the estimated antimicrobial activity of these proteins/peptides.

Materials and Methods

Antimicrobial Activity Assay

A radial diffusion assay (8) was used. Briefly, bacteria were grown overnight for 18 h at 37°C in trypticase soy broth (TSB). Midlogarithmic phase organisms were used by inoculating TSB with the growing bacteria for an additional 3 h at 37°C. The bacteria were centrifuged and washed in cold 10 mM sodium phosphate buffer (NAPB, pH 7.4) and resuspended in cold NAPB to achieve an optical density (620 nm) of 5×10^7 colony forming units (CFU)/mL. A volume containing 1×10^6 bacterial CFU was added to 10 mL of melted sterile agarose (10 mM NAPB, 3 mg TSB, 1% w/v of low electroendosmosis-type agarose, and .02% v/v Tween 20). After vortexing the bacteria with the agar, the agar was poured into a 100 × 15-mm petri dish.

A 3-mm-diameter gel punch was used to make 12 evenly spaced wells in solidified agar medium. The punch was sterilized between plates to prevent surface contamination. Five µL of test or control fluid was added to each well. The plates were incubated for 3 h at 37°C, and then overlaid with 10 mL of sterile agar (6% w/v TSB and 1% w/v low electroendosmosis-type agarose).

After a 12-h incubation at 37°C, the diameter of the clear zone surrounding the wells was measured with a 10 × measuring magnifier that contained a metric scale scribe in .1-mm increments. The diameter of clearing (clearance zone) was expressed in units (.1 mm = 1 U) minus the diameter of the well.

Evaluation of the Sensitivity and Precision of the Antimicrobial Activity Assay

Each protein/peptide and the control antibiotic was added to the diluent of choice (acetic acid or digesta) in 5-serial two-fold dilutions (Table 1). These dilution concentrations were determined to give a linear change in the total diameter clearance.

A regression equation was developed from the serial dilutions by plotting the known concentrations of a protein/peptide that was added to the fluid versus the respective clearance zones. The regression equation was then used to determine the minimum inhibitory concentration (MIC) for each protein/peptide tested.

The intra-assay variations for MIC estimates were determined by analyzing three samples of each protein/peptide/antibiotic dilution and determining the MIC from each sample (three MIC values/day for each protein/peptide/antibiotic). The inter-assay variation was determined by analyzing the three samples of each protein/peptide dilution on each of four consecutive days.

Bacteria

Two antibiotic-sensitive bacteria, a gram-negative (*Escherichia coli*) and a gram-positive (*Staphylococcus aureus*) bacteria were used to evaluate the efficacy of activity of the selected proteins/peptides. In addition, an antibiotic-resistant bacterium (*Escherichia coli* – nalidixic acid resistant) was evaluated.

Thad Stanton of the National Animal Disease Center (Ames, IA) donated the antibiotic susceptible strains of *E. coli* and *S. aureus*. Karl Dawson of Alltech (KY) donated the non-isogenic *Escherichia coli* – nalidixic acid resistant strain. Prior to usage, the bacteria were stored in an -80°C freezer.

When using the nalidixic acid-resistant bacteria, 80 µg of nalidixic acid was added to the culture tubes during growth, and 2 µg of nalidixic acid was added to each 10-mL tube of low electroendosmosis-type agarose.

Animal Digesta Collection and Recovery

Digesta fluid was collected from 250-pound pigs killed at the Iowa State University Meat Lab. Prior to slaughter, the pigs had been self-fed a fortified corn-soybean meal diet and were removed from feed 2 h prior to digesta collection. The pigs used in the collection had not been administered (orally or injected) antimicrobial agents for an extended length of time. The fluid was collected from the duodenum and jejunum of the pig and pooled into one sample. The contents were centrifuged at 10,000 × g for 20 min. The supernatant was collected and then centrifuged again. This procedure was repeated one additional time. The supernatant was then placed in a sterile syringe and filtered through a series of filters (5, .45, and .2 µm) and then placed into sterile polypropylene tubes.

For analysis of the activity of the proteins/peptides/antibiotic in the digesta fluid, the proteins/peptides/antibiotic were added to the digesta at a known level prior to centrifugation. The antimicrobial activity from the digesta was then compared with the same protein/peptide/antibiotic activity in acetic acid. To ensure that the peptide activity was not diminished during processing, known concentrations of each protein/peptide/antibiotic was added to processed digesta fluid and then tested for antimicrobial activity compared with the other digesta samples.

Proteins/Peptides/Antibiotic

Lactoferrin was received from DMV International Nutritionals (Fraser, NY). Lactoferricin B and the alpha-lactalbumin peptide, LDT2 (9), were synthetically developed by Biopeptide Co., LLC (San Diego, CA). Hen egg lysozyme was purchased from Sigma (St. Louis, MO), as well as, the antibiotic control, polymyxin B.

Efficacy of AMP were evaluated for their MIC *in vitro* when placed in acetic acid, acetic acid with various changes reflecting that in the digesta (e.g., pH and mineral composition), and in swine digesta.

Effects of components of Animal Digesta on Antimicrobial Activity of AMPs

Cationic minerals. The effect of the concentrations of six cationic minerals (Zn, Mg, Cu, Fe, Na, and K) on the antimicrobial activity of three proteins/peptides (lactoferrin, lactoferricin, and lysozyme) on *E. coli* (antibiotic-sensitive) were evaluated. Each protein/peptide was evaluated in acetic acid at the highest concentration used in the MIC determination.

Zn (Zn oxide), Mg (Mg oxide), Cu (Cu sulfate), and Fe (Fe oxide) were individually added to achieve six concentrations of each mineral (1,2, 3, 4, 5, and 6 mM). Na (NaCl) and K (KCl) were individually added to achieve concentrations of 20, 40, 60, 80, and 100 mM. Typical total and free ion concentrations in the small intestine of the pig are given in Table 3 (3).

pH. The effect of pH on the antimicrobial activity of three proteins/peptides (lactoferrin, lactoferricin, and lysozyme) on *E. coli* (antibiotic susceptible) were evaluated. Again, each protein/peptide was evaluated at the highest concentration used in MIC determination. The antimicrobial activity of each protein/peptide was evaluated in acetic acid and animal digesta with initial pHs of 4.4 and 6.5, respectively. The pH in acetic acid and digesta was lowered to 2 and 2.4, respectively, by the addition of 1 N hydrochloric acid (HCl).

Enzyme inhibition. The effect of protease enzyme inhibition in digesta fluid on the antimicrobial activity of three proteins/peptides (lactoferrin, lactoferricin, and lysozyme) on *E. coli* (antibiotic-susceptible) were evaluated. Again, each protein/peptide was evaluated at the highest concentration used in MIC determination. The antimicrobial activity of each protein/peptide was evaluated through the addition of Pepstatin A, an protease enzyme inhibitor from Sigma, to digesta samples prior to any centrifugation process. Pepstatin A was added at a concentration of 200 µg/mL digesta fluid.

Results and Discussion

Antimicrobial Assay Sensitivity and Variation

The antibiotic polymyxin B was initially used to determine the sensitivity and precision of the antimicrobial activity assay. The MIC value for polymyxin B was detected to be .76 ± .17 µg/mL. The intra- and inter-assay variation for MIC determination was .18 and .2%, respectively.

Antimicrobial Efficacy of Proteins/Peptides in In vitro

The use of antimicrobial proteins/peptides in the *in vitro* assay verified that these products do in fact kill selected bacteria (Table 2), and that AMPs possess the ability to kill antibiotic-resistant bacteria. MIC values for each protein/peptide are presented in Table 2.

The antibiotic-resistant strain of *E. coli* required AMP concentrations 10 to 15 times higher than that of the nonantibiotic-resistant strain. However, the two *E. coli* strains are not isogenic, which implies that the nalidixic acid-resistant bacteria has a different genetic makeup than the susceptible bacteria and has the potential to possess a unique response to the proteins/peptides. The greater concentration of the proteins/peptides needed to kill antibiotic-resistant bacteria could be due to some external membrane changes that reduce protein/peptide binding (e.g. alteration in molecular charges or decrease in certain fatty acids required for AMP binding).

The fact that not all of the peptides work on gram-positive bacteria is indicative of a similar change in membrane structure that is associated with the antibiotic resistant bacteria. This type of hypothesis is obvious when evaluating the antimicrobial activity of the alpha-lactalbumin peptide on the resistant strain of *E. coli*. Although the peptide does not work on the other bacteria, it does produce a killing effect on the resistant *E. coli*. This could be due to a membrane alteration that allows the peptide to bind and lyse the bacterium.

In general, the MIC values achieved in the researchers' laboratory were slightly lower than the literature values (1-2, 4-6, 9-11). The increase in antimicrobial activity could be associated with the characteristics of each protein or peptide evaluated, because the isolation procedures used can have an impact on activity due to differences in peptide configuration or presence of additional components, such as iron.

Overall, the intra-assay variation values for the lactoferrin, lactoferricin, and lysozyme were 8.7, .21, and 2.6%, respectively. The inter-assay variation values for the lactoferrin, lactoferricin, and lysozyme were 10.4, .70, and 2.3%, respectively.

Due to the fact that the alpha-lactalbumin peptide LDT2 had no effect on *E. coli* in the MIC test, it was not evaluated for the mineral, pH, or digesta fluid tests. The possible reasons that LDT2 did not work against either *E. coli* or *S. aureus* is that the peptide has been reported to only work against gram-positive bacteria (*S. aureus*); and that recent evidence has indicated that the peptide, as well as its protein precursor, must be present in at least dimer formation. Because the peptide was synthesized and purified, it is most likely not in dimer formation.

Effects of Components in Intestinal Digesta on Antimicrobial Efficacy of Proteins/Peptides In vitro

Overall, the various minerals added to acetic acid either had no effect on lactoferrcin or lysozyme; however, there was reduced antimicrobial activity of the protein, lactoferrin (Table 3). In addition, as the Zn concentration increased, the lactoferrin antimicrobial activity returned to a level similar to that of the acetic acid control.

Other literature has indicated a positive effect of cationic minerals on increasing antimicrobial activity of lactoferricin (1). The increase in antimicrobial activity

that Bellamy et al. (1) discusses could be due to the fact that proteins/peptides are drawn to the cation/anion charges of certain minerals.

Upon exposing the peptides to a pH of 2 (similar to that of the stomach), the activity of the proteins/peptides was reduced to approximately 67% of that of a pH of 7 (Table 4). This response would be expected, because the lowering of the pH should alter the charges on various amino acids, which would change the proteins/peptides ability to bind bacteria. Kuwata et al. (7) demonstrates that lactoferrin can survive the pH of the human stomach; however, lactoferrin is cleaved by digestive enzymes during this process. Since some of the lactoferrin and its activity is retained in the study, one would assume that the disulfide bridges aid in preventing the proteins/peptides from becoming denatured when exposed to a lower pH.

The addition of the antimicrobial proteins/peptides to digesta fluid created antimicrobial activity greater (up to twice as great) than the values given in Table 2 (Table 5). However, digesta fluid without the addition of AMPs had no antimicrobial activity.

One would expect the increase in activity with a protein that would be degraded into its more active peptide through pepsin and trypsin cleavage; however, this increase in antimicrobial activity was also associated with the peptides which have already been cleaved. The increase antimicrobial activity would indicate that there are additional factors within the digesta fluid (possibly other trace minerals or a synergistic effect with a combination of minerals, digesta peptides not filtered out) that enhance the antimicrobial activity of the proteins/peptides.

The inhibition of digestive enzymes did not alter the increase in protein/peptide activity compared with the activity of these products in untreated digesta fluid. This result would support the previous comment in that the change in activity is not entirely due to an enzymatic cleaving of the protein that would result in higher antimicrobial activity.

Because the addition of selected minerals did not affect the antimicrobial activity of these proteins/peptides, and in some cases, slightly decreased activity, one would assume that the increase in activity in the digesta was not associated with the mineral composition of the digesta or the respective corn-soybean meal diet the pigs were fed prior to slaughter.

The fact that the nutrient concentration of certain minerals in the digesta, digestive enzymes, and pH did not alter the antimicrobial activity in the *in vitro* studies indicates that other factors likely are contributing to the enhanced antimicrobial activity of the proteins/peptides in animal digesta.

In conclusion, the antimicrobial radial diffusion assay has been shown to be a highly sensitive and precise assay for evaluated antimicrobial activity with low intra- and inter-assay variation. Furthermore, natural proteins/peptides from milk and eggs have been shown to elicit antimicrobial activity against antibiotic-susceptible and -resistant bacteria both in acetic acid and digesta fluid media.

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Table 1. Concentration (μg/mL) of each protein/peptide used for analysis of antimicrobial assay variation and sensitivity.

Protein/Peptide/Antibiotic	Concentration (μg/mL)
Lactoferrin	3000
	1500
	750
	375
	188
Lactoferricin	100
	50
	25
	12.5
	6.25
Lysozyme	500
	250
	125
	62.5
	31.25
Alpha-lac. LDT2	100
	50
	25
	12.5
	6.25
Polymyxin B	2400
	1200
	600
	300
	150

Table 2. Minimum inhibitory concentrations (MIC, μg/mL) of an antibiotic and antimicrobial proteins/peptides *in vitro*.

		Antibiotic	Natural Proteins/Peptides			
Bacteria	Antibiotic Status	Polymyxin B	Lactoferrin	Lactoferricin	LDT2	Lysozyme
E. coli	Susceptible	.76 ± .17	6.4 ± 1.2	2.15 ± .34	No effect	7.06 ± .68
E. coli	Resistant	.76 ± .18	100 ± 13.1	20 ± 2.2	250 ± 18.6	100 ± 15.3
S. aureus	Susceptible	.90 ± .20	No effect	2.10 ± .34	No effect	No effect

Range tested found to be linear.
Mean of 12 samples ± SEM.

Table 3. Effects of mineral concentrations on antimicrobial activity of proteins/peptides. Values reported as diameter of clearing expressed in units (.1 mm = 1 U) with standard errors.

Mineral (mM)			Lactoferrin	Lactoferricin	Lysozyme
Acetic acid	Total in digesta	Free in digesta	(3,000 μg/mL)	(50 μg/mL)	(250 μg/mL)
Acetic acid control			360 ± 21	550 ± 19	580 ± 33
Zn	.710	.11			
1			140 ± 10	50 ± 0	650 ± 0
2			200 ± 0	530 ± 30	650 ± 0
3			275 ± 25	530 ± 10	600 ± 50
4			290 ± 10	510 ± 10	600 ± 50
5			335 ± 25	510 ± 0	570 ± 30
6			310 ± 10	525 ± 5	585 ± 35
Mg	15.7	6.4			
1			125 ± 5	515 ± 5	510 ± 40
2			205 ± 5	540 ± 0	570 ± 70
3			245 ± 5	530 ± 0	640 ± 0
4			335 ± 55	555 ± 5	645 ± 95
5			305 ± 5	555 ± 5	705 ± 25
6			310 ± 10	530 ± 10	665 ± 15
Fe	2.33	.02			
1			50 ± 15	555 ± 5	645 ± 5
2			50 ± 10	530 ± 10	655 ± 5
3			155 ± 5	545 ± 5	640 ± 0
4			145 ± 5	560 ± 0	625 ± 5
5			120 ± 0	565 ± 15	625 ± 5
6			145 ± 15	565 ± 5	635 ± 5
Ca	46.1	5.2			
1			145 ± 5	540 ± 0	575 ± 25
2			150 ± 10	565 ± 15	550 ± 20
3			155 ± 5	555 ± 5	625 ± 25
4			145 ± 5	530 ± 10	615 ± 35
5			150 ± 0	560 ± 10	650 ± 50
6			170 ± 10	555 ± 5	660 ± 10
NaCl[a]	138	116.8			
20			100 ± 0	530 ± 10	625 ± 25
40			85 ± 15	545 ± 5	540 ± 10
60			90 ± 0	525 ± 5	560 ± 10
80			95 ± 5	555 ± 5	545 ± 5
100			100 ± 0	530 ± 10	565 ± 15
KCl[a]	30.4	26.7			
20			120 ± 10	535 ± 10	540 ± 25
40			90 ± 15	550 ± 5	580 ± 15
60			100 ± 10	540 ± 10	560 ± 10
80			110 ± 5	545 ± 15	555 ± 0
100			95 ± 0	550 ± 10	550 ± 5

[a] NaCl and KCl levels for digesta are reported as Na and K ions, not the salt concentrations
Values reported as means ± SEM.

Table 4. Effect of digesta fluid on antimicrobial activity of proteins/peptides and an antibiotic. Values reported as diameter of clearing expressed in units (.1 mm = 1 U) and percentage of antimicrobial activity in digesta relative to that in acetic acid control.

Antibiotic/ Protein/Peptide	(μg/mL)	Assay Media		Relative Antimicrobial Activity
		Acetic Acid	Digesta Fluid	
Lactoferrin	6000	250 ± 0	1,097 ± 3.3	439
Lactoferricin	50	300 ± 0	710 ± 5.8	237
Lysozyme	250	690 ± 0	817 ± 8.8	118
Polymyxin B	1200	750 ± 0	240 ± 13.3	32

Means reported from three samples ± SEM.

Table 5. Effect of pH on antimicrobial activity of proteins/peptides and an antibiotic. Values reported as diameter of clearing expressed in units (.1 mm = 1 U) and percentage antimicrobial activity in digesta relative to that in acetic acid control.

Antibiotic/ Protein/Peptide	(μg/mL)	Acetic Acid			Digesta Fluid		
		pH 4.4	pH 2	% Activity	pH 6.5	pH 2.4	% Activity
Lactoferrin	6000	250 ± 0	177 ± 26.7	71	1097 ± 3.3	393 ± 23.3	36
Lactoferricin	50	300 ± 0	150 ± 0	50	710 ± 5.8	323 ± 8.6	45
Lysozyme	250	690 ± 0	603 ± 3.3	87	817 ± 8.8	710 ± 13.3	87
Polymyxin B	1200	750 ± 0	673 ± 13.3	90	240 ± 13.3	240 ± 0	100

Means reported from three samples ± SEM.